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Endotoxin induces rat lung ribonuclease activity

Linda B. Clerch, Angelique Wright and Donald Massaro

The Lung Biology Laboratory, The Departments of Pediatrics and Medicine, Georgetown University Medical Center, Washington, DC 20007, USA

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Lipopolysaccharide (endotoxin), a component of Gram-negative bacteria, causes marked alterations in eukaryotic gene expression and cellular physiology. We show that within one hour of injection of endotoxin into adult rats there is an induction of ribonuclease activity in the lung. The degradation of RNA was prevented by treatment of the lung extract from endotoxin-injected rats with ribonuclease inhibitor (RNasin). We suggest that induction by endotoxin of ribonuclease activity is a novel mechanism by which cells could alter gene expression to meet an environmental challenge and caution that the presence of ribonuclease can hinder molecular biological analyses of tissue extracts from endotoxin-treated rats

Ribonuclease; Endotoxin; Lipopolysaccharide; RNA-binding; Rat lung

1. INTRODUCTION

Lipopolysaccharide (endotoxin) is the component of the wall of Gram-negative bacteria responsible for the injurious, frequently lethal sequelae associated with infections by these organisms [1]. Its interaction with eukaryotic cells constitutes a major environmental challenge that results in remarkable changes in cellular physiology and gene expression. In the present study we show that within one hour of injecting endotoxin into adult rats there is induction of ribonuclease (RNase) activity. We suggest that induction of ribonuclease activity by endotoxin is a mechanism by which cells could alter gene expression to meet an environmental challenge. In addition, the endotoxin-induced RNase activity eliminated the ability to detect catalase RNA–protein binding activity in lung extracts. This latter observation points to an important potential difficulty in using tissue samples from endotoxin-treated animals in which an induced RNase interferes with molecular biological analyses.

2. MATERIALS AND METHODS

2.1. *Animal studies*

Adult Sprague–Dawley rats (200–250 g) were obtained from Zivic Miller Inc. Zelienople, PA, and maintained in the animal care facility at Georgetown University Medical Center in accordance with institutional guidelines. Rats were injected intraperitoneally with endotoxin (500 μg/kg, lipopolysaccharide from *Salmonella typhimurium*) in 0.15 M NaCl (saline). Control rats were injected with an equal volume of saline. Rats were killed by exsanguination after being brought to surgical levels of anesthesia with pentobarbital sodium (> 80 mg/kg). Their lungs were perfused in situ with saline, excised, frozen in liquid nitrogen, and stored at −70°C. We obtained cellular extract from excised rat lungs by using a Brinkman polytron to homogenize the lungs in 25 mM Tris-HCl buffer, pH 7.0, containing 0.1 mM EDTA, 1% Triton X-100, 40 mM KCl, 0.2 U/μl aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Lung homogenate was centrifuged at 12,000 × *g* for 10 min; the supernatant fluid was collected and its protein concentration measured using Coomassie Plus assay reagent from Pierce with bovine serum albumin as a standard. Samples of the 12,000 × *g* supernatant material were frozen at −70°C until used.

Correspondence address: L.B. Clerch, Lung Biology Laboratory, PSB, GM-12, Georgetown University Medical Center, 3900 Reservoir Road N.W., Washington, DC 20007, USA. Fax: (1) (202) 687 8538.

2.2. *Filter binding assay for ribonuclease activity*

Radiolabeled catalase cRNA was generated by in vitro transcription with [^{32}P]CTP as previously described [2]. ^{32}P-labeled catalase RNA probe was incubated with lung extract in 15 μl of buffer containing 5% glycerol, 40 mM KCl, 3 mM $MgCl_2$ and 10 mM HEPES (pH 7.6). After a 30 min incubation at 25°C, 1 ml of 0.3 M NaCl, 30 mM NaAcO, 3 mM ZnAcO, and 100 μg/ml salmon testes DNA was added, followed by 0.1 ml of 100% of TCA. After mixing and incubating on ice for 15 min, the samples were filtered onto 2.5 cm Whatman GF/c filters, washed twice with 3% TCA, 1% $NaPP_i$, and once with 95% ethanol. The radioactivity on the filters was quantitated by scintillation counting in optifluor-o (Packard). Data were expressed as dpm of TCA-precipitated probe as a function of lung extract protein.

2.3. *Assay for catalase RNA-binding protein*

To explore the potential sequelae of the induction of the RNase, we used a gel retention assay to assess the binding of lung protein to a catalase cRNA probe as described previously [2]. Briefly, ^{32}P-labeled rat catalase cRNA (10,000 dpm) was incubated with rat lung extract (30 μg protein) in 15 μl of buffer containing 5% glycerol, 40 mM KCl, 3 mM $MgCl_2$ and 10 mM HEPES (pH 7.6). After a 30 min incubation at 25°C, 1U of RNase T1 (BRL) was added and incubation allowed to continue for 15 min; heparin sulfate was then added to a concentration of 6 μg/μl and the incubation continued for an additional 15 min. The binding reaction mixtures were mixed with 1 μl of 97% glycerol, 1% Bromophenol blue and subjected to electrophoresis on a low-crosslinked 4% polyacrylamide gel (acrylamide/bisacrylamide 60:1) containing 89 mM Tris borate (pH 8.0) and 2 mM EDTA. Electrophoresis was carried out at 14 V/cm; the gel was then transferred to filter paper, dried and subjected to radioautography at −70°C with an intensifying screen

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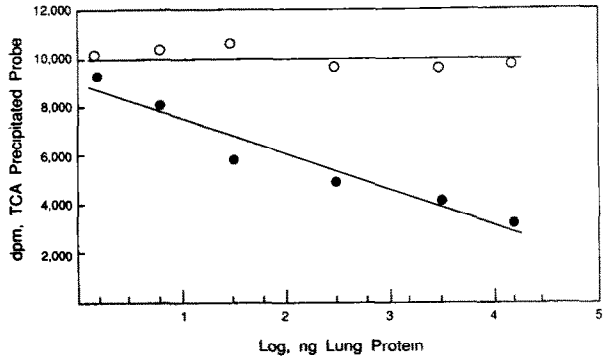

Fig. 1. Lung ribonuclease activity is induced by endotoxin treatment of adult rats. In a filter-binding assay, ^{32}P-labeled catalase cRNA was incubated for 30 min at 25°C with lung extract from adult rats killed one hour after intraperitoneal injection of endotoxin (500 μg/kg lipopolysaccharide from *Salmonella typhimurium*). After incubation, trichloroacetic acid (TCA) was added to a final concentration of 10%, the samples were filtered onto Whatman GF/C filters and counted in optifluor-o (Packard). Data are expressed as dpm of TCA-precipitated probe as a function of lung extract protein (log ng). Open circles show the protection against loss of TCA-precipitable probe when lung extract from endotoxin-treated rats was preincubated for 30 min at 25°C with 40 U of RNase inhibitor, RNasin (Promega); solid circles show the loss of TCA-precipitable probe when lung extract from endotoxin-treated rats was preincubated in the same manner but without the RNase inhibitor.

3. RESULTS AND DISCUSSION

Incubation of lung extract from endotoxin-treated rats with ^{32}P-labeled catalase cRNA (probe) resulted in a dose-dependent loss of trichloroacetic acid (TCA) precipitable probe in a filter binding assay (slope = 0.253, μg protein vs. TCA precipitable dpm). Preincubation of these lung extracts with the RNase inhibitor, RNasin (Promega), completely blocked loss of the probe (Fig. 1). When extract from saline-treated rats was used in the filter binding assay, we did not detect any RNase activity (slope = + 5.7, μg protein vs. TCA-precipitable dpm). These results indicate that endotoxin treatment activated or induced the synthesis of an RNase that is inhibitable by RNasin, an RNase inhibitor of eukaryotic RNases of the neutral type; RNasin inhibits RNases A, B and C but not RNase T1, S1 nuclease, RNase from *Aspergillus* or RNase H [3].

Rat lung contains a protein that interacts specifically with catalase mRNA to form RNA-protein redox-sensitive complexes [2]. The endotoxin-induced RNase activity was manifest as a loss of catalase mRNA-protein binding due to the degradation of the ^{32}P-labeled RNA probe used in the gel shift assay. This loss of probe and its retention by preincubation with RNasin was associ-

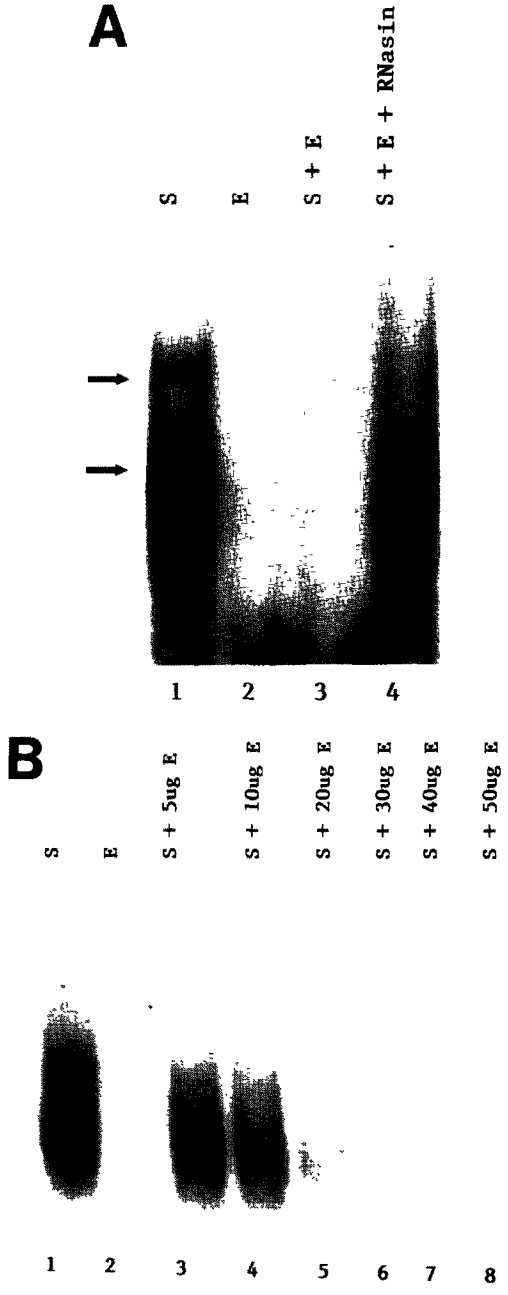

Fig. 2. Elimination of rat lung catalase RNA–protein binding by endotoxin treatment is prevented by RNasin. (A) We used a gel mobility shift assay to assess the binding of lung protein to a catalase cRNA probe. Radioautography demonstrated RNA–protein complexes (arrows) were present when 30 μg of lung protein extract from saline-treated rats was incubated with ^{32}P-labeled catalase cRNA probe (S, lane 1) but were absent when 60 μg lung extract protein from adult rats given endotoxin (500 μg/kg) was assayed (E, lane 2). Complex formation was eliminated when lung extract from saline-treated rats was pretreated for 30 min at 25°C with 60 μg of protein extract from endotoxin-treated rats (S + E, lane 3). The endotoxin-induced inhibition of complex formation was blocked by pre-incubation (30 min at 25°C) of the lung extract from endotoxin-treated rats with 40 U of RNase inhibitor, RNasin (S + E + RNasin, lane 4). (B) Catalase RNA– protein complexes were formed when 30 μg of lung extract protein from a saline-treated adult rat was used in the gel mobility shift assay (S, lane 1). Complex formation was absent between catalase RNA and lung extract from an adult rat given endotoxin (500 μg/kg) one hour before being killed (E, lane 2). Complex formation was eliminated in a dose–response manner when the binding reaction was performed after pretreatment of 30 μg S extract protein with 5, 10, 20, 30, 40 and 50 μg of E extract protein, lanes 3 to 8, respectively.

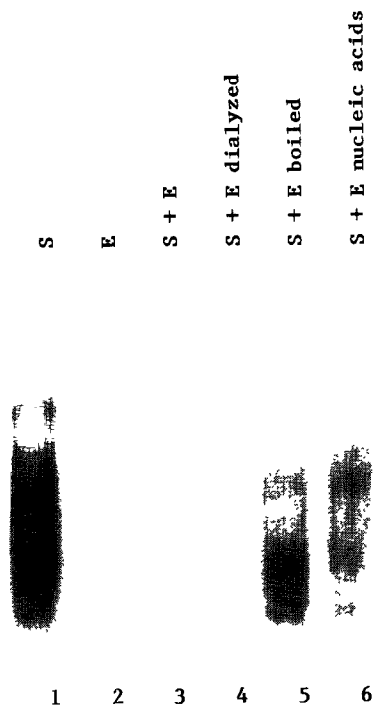

Fig. 3. Characterization of the elimination of rat lung catalase RNA–protein binding by endotoxin treatment. Catalase RNA–protein complexes were present when lung extract from a saline-treated adult rat was used in the gel shift assay (lane 1) but were absent when lung extract from an endotoxin-treated rat was used (lane 2). Lung extract protein (30 μg) from a saline-treated rat was pretreated for 30 min at 25°C with 60 μg of lung extract protein from endotoxin-treated rat (lane 3), endotoxin-extract dialyzed using dialysis tubing with a cut-off range of 6000–8000 molecular weight (lane 4), endotoxin extract boiled for 5 min (lane 5), or endotoxin extract treated with phenol/chloroform to isolate the nucleic acid fraction (lane 6).

ated with the absence and presence, respectively, of catalase mRNA–protein complexes (Fig. 2A). Furthermore, when lung extracts from saline-injected rats were incubated with extracts from endotoxin-injected rats, complex formation could be eliminated in a dose-dependent manner (Fig. 2B). This inhibitory action on RNA–protein complex formation of lung extract from endotoxin-treated rats was not lost by dialysis (6000–8000 M_r cut-off) but was lost by boiling (Fig. 3). The nucleic acid fraction of the extract from endotoxin-treated rats did not cause inhibition of the binding activity indicating that a ribozyme is not likely to be responsible for the RNase activity (Fig. 3). From these data we conclude endotoxin treatment induces ribonuclease activity; a consequence of RNase induction is an interference with the ability to test for catalase RNA-binding protein in lung extract from endotoxin-treated rats unless a RNase inhibitor is included in the binding reaction. This observation indicates caution must be exercised when using tissue extract from endotoxin-treated rats in molecular biological analysis.

From a physiological point of view, we suggest that the induction of endotoxin of ribonuclease activity is a mechanism by which cells could alter gene expression to meet an environmental challenge. That selective-ribonuclease induction by endotoxin may provide a mechanism to respond to environmental challenges is supported by the observation that in rats endotoxin treatment causes a rapid fall in the concentration of hepatic albumin mRNA but an increase in the hepatic mRNAs for two acute-phase proteins, α1-acid glycoprotein and α2-macroglobulin [4]. It will be important to determine if induction of ribonuclease activity is a general mechanism to selectively modulate gene expression by enhancing synthesis of proteins needed to meet bacterial and other environmental challenges.

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